SIGNIFICANCE OF ZONE 2 PEAK ON CAPILLARY ELECTROPHORESIS FOR THE DETECTION OF HAEMOGLOBIN CONSTANT SPRING

BY

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LIST OF ABBREVIATIONS

ARMS	Multiplex amplification refractory mutation systems
CE	Capillary electrophoresis
CS	Constant Spring
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra-acetic acid
FBC	Full blood count
$--^{FIL}$	Alpha zero thalassaemia Filipino type deletion
FSC	Forward scattered light
Hb	Haemoglobin
HCT	Haematocrit
HPLC	High performance liquid chromatography
HRPZ II	Hospital Raja Perempuan Zainab 2
HUSM	Hospital Universiti Sains Malaysia
IMR	Institute of Medical Research
MCH	Mean cell haemoglobin
MCHC	Mean cell haemoglobin concentration
MCV	Mean cell volume
$--^{MED}$	Alpha zero thalassaemia Mediterranean type deletion

MLPA	Multiplex ligation-dependent probe amplification
mRNA	Messenger ribonucleic acid
OFT	Osmotic fragility test
PCR	Polymerase chain reaction
RBC	Red blood cell count
RDW	Red cell distribution width
RNA	Ribonucleic acid
$--^{SEA}$	Alpha zero thalassaemia South-east Asian type deletion
SFL	Side fluorescence light
SLS	Sodium lauryl sulphate
SSC	Side scattered light
$--^{THAI}$	Alpha zero thalassaemia Thailand type deletion
WHO	World Health Organization
α	Alpha
α^{+}	Alpha plus (reduced production of alpha chain from the affected chromosome)
α°	Alpha zero (no alpha chains produced)
$\alpha\text{-}^{3.7}$	Alpha plus thalassaemia 3.7 deletion
$-\alpha^{4.2}$	Alpha plus thalassaemia 4.2 deletion
α^{CS}	Alpha Constant Spring

$\alpha^T\alpha$	Mutations of the alpha2 gene
$\alpha\alpha^T$	Mutations of the alpha1 gene
β	Beta

KEPENTINGAN KEHADIRAN PUNCAK PADA ZON 2 KAPILARI ELEKTROFORESIS (CE) DALAM MENGESAN KEWUJUDAN HEMOGLOBIN CONSTANT SPRING.

ABSTRAK

Hemoglobin Constant Spring adalah defek pada titik mutasi bagi α-talasemia di "termination codon" dan menyebabkan penyambungan pada rantaian α dan mengurangkan kestabilan mRNA. Ia merupakan α-talassemia jenis "non deletional" yang biasa dan lazim dijumpai di kebanyakan negara-negara Asia Tenggara. Hemoglobin yang abnormal ini mempunyai kepelbagaian dan variasi dari aspek klinikal dan boleh berinteraksi dengan α° dan menyebabkan kewujudan Hemoglobin H- Constant Spring.

Pemeriksaan hemoglobin yang abnormal ini dilakukan menggunakan kapilari elektroforesis. Kapilari elektroforesis ini merupakan salah satu teknik pemisahan analisis dan boleh digunakan untuk memisahkan dan mengkuantitatifkan Hemoglobin A_2, Hemoglobin F dan hemoglobin abnormal yang lain. Ia adalah kaedah yang sensitif untuk mengesan kewujudan Hemoglobin Constant Spring di mana ia akan memberikan kehadiran puncak pada zon 2 di kapilari elektroforesis.

Tujuan kajian ini dilakukan adalah untuk menentukan kepentingan kehadiran puncak pada zon 2 kapilari elektroforesis dalam mendiagnosis Hemoglobin Constant Spring, membandingkan profil hematologi di antara genotip Hemoglobin Constant Spring yang berlainan dan juga untuk menentukan lingkungan nilai di zon 2 pada kapilari elektroforesis untuk genotip Hemoglobin Constant Spring yang berlainan.

Satu kajian keratan rentas telah dilakukan daripada Julai 2015 sehingga Julai 2016 dengan mengumpul secara rawak sebanyak 137 sampel yang menunjukkan kehadiran puncak pada zon 2 kapilari elektroforesis. Sampel-sampel ini telah diuji untuk parameter-parameter sel darah merah (Hb, RBC, MCV,MCH dan RDW) dengan menggunakan automasi Sysmex XN 3000,"Sysmex Corporation, USA. Analisis hemoglobin dilakukan menggunakan kapilari elektroforesis (Sebia CAPILLARYS2 Flex Piercing Sebia, PN 1227, France) dan analisis DNA menggunakan "Multiplex PCR" dan "ARMS" dilakukan untuk "deletional" dan "non deletional" α talassemia.

Daripada 137 sampel, 118 sampel (86.1%) yang menunjukkan puncak pada zon 2 kapilari elektroforesis telah diuji positif untuk mutasi pada "termination codon" Hemoglobin Constant Spring. Majoriti sampel yang diuji adalah jenis heterozigot Hemoglobin Constant Spring iaitu 92 (67.2%), diikuti oleh kompaun heterozigot iaitu 22 sampel (16%) dan homozigot iaitu sebanyak 4 sampel (2.9%).

Terdapat bilangan sampel yang menunjukkan puncak positif pada Zon 2 kapilari elektroforesis yang signifikan dan juga positif untuk mutasi pada "termination codon" Hemoglobin Constant Spring. Julat nilai Hemoglobin Constant Spring untuk genotip heterozigot adalah di antara min 0.67±0.27%. Manakala untuk julat nilai Hemoglobin Constant Spring bagi genotip homozigot dan kompaun heterozigot adalah masing-masing di antara 4.5 hingga 5.5 % dan 0.3 hingga 2.2%. Melalui kajian ini, terdapat perbezaan parameter hematologi yang ketara di antara ketiga-tiga genotip yang berlainan iaitu heterozigot, kompaun heterozigot dan homozigot Hemoglobin Constant Spring. Oleh itu, kita boleh membezakan ketiga-tiga kumpulan ini melalui parameter-parameter hematologi dan nilai Hemoglobin Constant Spring pada puncak zon 2 di kapilari elektroforesis sekiranya tiada analisis molekular disediakan atau tidak dapat diakses di mana-mana pusat/kemudahan.

SIGNIFICANCE OF ZONE 2 PEAK ON CAPILLARY ELECTROPHORESIS FOR THE DETECTION OF HAEMOGLOBIN CONSTANT SPRING

ABSTRACT

Haemoglobin Constant Spring (Hb CS) is a point mutational defect of α thalassaemia at the termination codon and leads to α chain extension and reduced mRNA stability. It is a prevalent and common non deletional α thalassemia in most Southeast Asian countries. This abnormal haemoglobin has clinical heterogeneity and can interact with α° and cause non deletional Hb H CS.

Screening for this abnormal haemoglobin is performed by capillary electrophoresis (CE). It is one of the analytical separation techniques and can be used to separate and quantitate Hb $A_{2,}$ Hb F and other abnormal haemoglobins. CE gives peak at the Zone 2 for Hb CS and it is a sensitive method to detect Hb CS.

The aims of this study were to determine the significance of presence of Zone 2 peak on CE in diagnosing Hb CS, to compare the haematological profiles between different Hb CS genotypes and to estimate range for Zone 2 peak by CE with different Hb CS genotypes.

A cross sectional study was done from July 2015 to July 2016 by collecting 137 samples randomly which showed positive peak on Zone 2 of CE. The samples were tested for red cell indices (Hb, RBC, MCV, MCH, and RDW) by using Sysmex XN 3000 analyzer, Sysmex Corporation, USA. Haemoglobin analyses were performed using CAPILLARYS2 Flex-Piercing System Sebia, PN 1227, France and DNA analyses using Multiplex PCR and ARMS were done for non deletional and deletional α thalassaemia.

One hundred and eighteen (86.1%) out of 137 samples that showed positive peak in Zone 2 of CE, were positive for termination codon Hb CS mutation, confirmed by molecular analysis. The most common types of Hb CS found was Heterozygotes Hb CS which was 92 samples (67.2%), followed by compound heterozygotes which was 22 samples (16%) and homozygotes group which was 4 samples (2.9%).

There were significant number of samples that had positive peak at Zone 2 which confirmed for termination codon CS mutation. The range of Hb CS level for Hb CS trait can be estimated to lie within mean of 0.67±0.27%. Meanwhile, the range of Hb CS level for compound heterozygotes Hb CS and homozygotes Hb CS were from 0.3 to 2.2 % and 4.5 to 5.5%, respectively. This might aid in making the diagnosis if the molecular technique is not available.

Significant haematological differences which include Hb level, MCV, MCH, RDW, RBC count and Hb CS level were observed between heterozygotes, homozygotes and compound heterozygotes Hb CS. Consequently, we will able to predict these groups from the haematological indices and Hb CS level/ quantification on zone 2 peak if the molecular technology is not available.

CHAPTER 1

GENERAL

INTRODUCTION

1.0 GENERAL INTRODUCTION

Haemoglobin (Hb) Constant Spring (CS) is a non deletional alpha (α) thalassaemia on α2 genes as a result of impaired RNA translation consequent on a termination codon mutation leading to an elongated messenger ribonucleic acid (mRNA) and α globin chain. This variant contains point mutation at the termination codon, in which the defect is (TAA>CAA) and it is associated with reduced mRNA stability and elongation of α chain. This point mutation leads to α chain extension up to another 31 amino acid residues (Laig *et al.*, 1990).

α thalassaemia is highly prevalent in Malaysia and its neighbouring Southeast Asian countries. It is a public health concern in Malaysia, with a gene frequency of 4.1%. On the other hand, Hb CS occurs with a frequency of 1-4% in most Southeast Asian countries and 1-6% in Malaysia. (Ahmad *et al.*, 2013)

The abnormal Hb in CS leads to unstable mRNA and cause decrease in rate of α globin chain synthesis. The heterozygote of Hb CS have normal clinical and haematological features but if compared to deletional alpha plus (α^+) thalassaemia, the heterozygous states of non deletional forms are sometimes associated with mild hypochromic anaemia. Meanwhile, the homozygotes show clinical presentation similar as thalassaemia intermedia with mild anaemia, jaundice and presence of hepatosplenomegaly. However the interaction of the Hb CS gene with deletional α thalassaemia is usually more severe than deletional Hb H disease in which some of the patients are dependent to transfusion (Liao *et al.*, 2010).

Screening for this abnormal haemoglobin is performed by capillary electrophoresis (CE). It is one of the analytical separation techniques that had been widely used in clinical

laboratories (Petersen *et al.*, 2003). The CE system can be used to separate and quantitate Hb $A_{2,}$ Hb F and other abnormal haemoglobin.

CE will give peak at the Zone 2 for Hb CS. Other common variant that shares the same peak as Hb CS is Hb Paksé. The incidence of Hb Paksé can also be observed at a significant prevalence but might have been underestimated. This is possibly due to misidentifying the cases as Hb CS (Viprakasit *et al.*, 2002) . Hb Paksé also has similar clinical presentation as Hb CS. However, the prevalence of this type of variant has not been described in Malaysia previously. Other rare variants that can give similar peak in Zone 2 of CE are Hb C, Hb F-Texas, Hb C-Harlem, variant de Hb A_2 "Setif" and other rare haemoglobins (Sebia, 2013).

This study was done to determine the significance of presence of Zone 2 peak on CE in diagnosing Hb CS. Since Hb CS is prevalent in this region and shows clinical heterogeneity, hence reliable detection of Hb CS by CE can be used for further management of patients with this type of haemoglobinopathy in centres that do not have access to molecular studies. In addition, we hope with this study, different types of Hb CS can be predicted if the molecular technique is not available.

CHAPTER 2

LITERATURE REVIEW

2.0 LITERATURE REVIEW

2.1 HAEMOGLOBIN GENETICS

A normal haemoglobin molecule is made up by haem and globin in which the former is responsible for oxygen transport while the latter serves to protect haem from oxidation. The synthesis of globin chain is characterized by two switches. As early as 5 weeks of gestation, the embryonic haemoglobin switches to fetal haemoglobin and the synthesis completed by week 10. Although expression of beta (β) globin occurs early, around 8 weeks but the β globin synthesis is still low with a high up regulation just before birth, correlating with a decrease in γ globin expression. This indicates the replacement of fetal haemoglobin by adult haemoglobin (A. Victor Hoffbrand, 2011) (as shown in Table 2.1).

Table2.1. Haemoglobins normally present during adult, fetal and embryonic periods of life

Haemoglobin species	Globin chains	Period when normally present
A	$\alpha_2\beta_2$	Major haemoglobin in adult life
A_2	$\alpha_2\delta_2$	Minor haemoglobin in adult life
F	$\alpha_2\ \gamma_2$	Minor haemoglobin in adult life, major haemoglobin in fetal life with a declining percentage through the neonatal period
Gower 1	$\zeta_2\varepsilon_2$	Significant haemoglobin during early intrauterine life
Gower 2	$\alpha_2\varepsilon_2$	Significant haemoglobin during early intrauterine life
Portland	$\zeta_2\ \gamma_2$	Significant haemoglobin during early intrauterine life

Adapted from (Bain, 2006).

The genes encoding the α and β chain are located on the chromosome 16 and chromosome 11 respectively. *Cis*-acting enhancer sequences control the tissue specific expression, which is located upstream at each gene cluster (Steinberg *et al.*, 2009). Only a single β gene is present while two α genes are present on each chromosome. Thus, it generates more complex hereditary patterns (Giordano, 2013). The synthesis of α and β globin chains take place in erythroid precursors to the reticulocyte stage (Higgs, 1993).

2.2 INTRODUCTION OF THALASSAEMIA

2.2.1 Definition

Thalassaemia: an inherited heterogeneous disorder of haemoglobin synthesis that results in a diminished rate of synthesis of the haemoglobin or haemoglobins (Bain, 2006).

α thalassaemia: a group of thalassaemias characterized by absent or reduced rate of α globin chain production, commonly resulting from the deletion of one or more of the α globin genes, less often it results from the mutation of locus control genes or genes encoding *trans*-acting factors or altered structure of an α gene (Bain, 2006).

Haemoglobinopathy: an inherited disorder resulting from the synthesis of a structurally abnormal haemoglobin or also can be used to include group of thalassaemias with reduced rate of synthesis of one of the globin chains (Bain, 2006).

2.2.2 Background and incidence of α thalassaemia

The name of 'thalassaemia' was given by Whipple and Bradford in 1936. It is derived from Greek word, sea. In 1925, Cooley and Lee were the first person that described this condition (Weatherall *et al.*, 2006). Four α globin genes present in normal individuals and they are linked as pairs, α2 and α1, at the tip of each chromosome 16(A. Victor Hoffbrand, 2016). In α thalassaemia, it is commonly result from the deletion of a large part or the entire gene. Non deletional α thalassaemia also can occur as a result from the mutations of the α2 gene ($\alpha^T\alpha$ thalassaemia) or the α1 gene ($\alpha\alpha^T$ thalassaemia). Hence, α thalassaemia can be divided broadly into deletional and non deletional thalassaemia (Harteveld and Higgs, 2010).

α thalassaemia also can be classified as alpha zero (α°) thalassaemia in which no α chains are produced from both linked pairs or reduced production of α chain from the affected chromosome, known as α^+. The α° thalassaemia are caused by deletion of both α globin genes and the deletions vary in size and commonly associated with geographical area (as shown in Figure 2.1). In Southeast Asia, the commonest α° are alpha zero Southeast Asian type deletion ($--^{SEA}$) and alpha zero thalassaemia Mediterranean type deletion ($--^{MED}$) (Harteveld and Higgs, 2010).

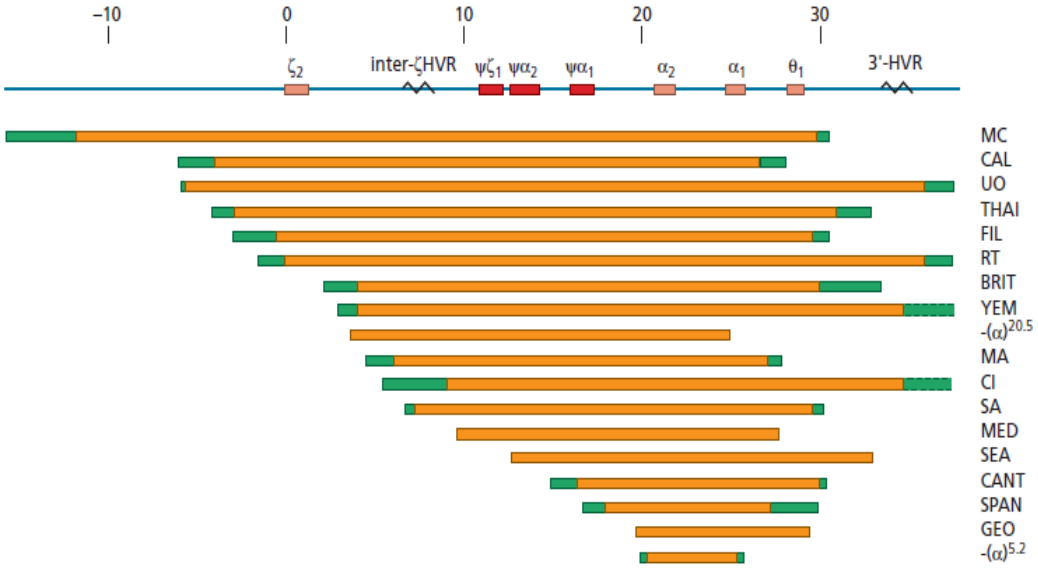

Figure 2.1. The α globin gene cluster deletions that underlie α° thalassaemia. The yellow colour represent extent of each deletion and green colour represent regions of uncertainty. Adapted from (A. Victor Hoffbrand, 2016).

Deletion of one of the normal four α globin genes will result in α^{+} thalassaemia (-α/αα). There are three homologous sub-segments which are referred as X, Y and Z (as shown in Figure 2.2).The commonest α^{+} thalassaemia in Malaysia are α plus thalassaemia 3.7 ($\alpha\text{-}^{3.7}$) and α plus thalassaemia 4.2 ($-\alpha^{4.2}$). $\alpha\text{-}^{3.7}$ is the rightward deletion in which there is misalignment and recombination between the Z segments while $-\alpha^{4.2}$ is leftward deletion in which there is crossover between the X boxes which are 4.2kb apart (A. Victor Hoffbrand, 2016).

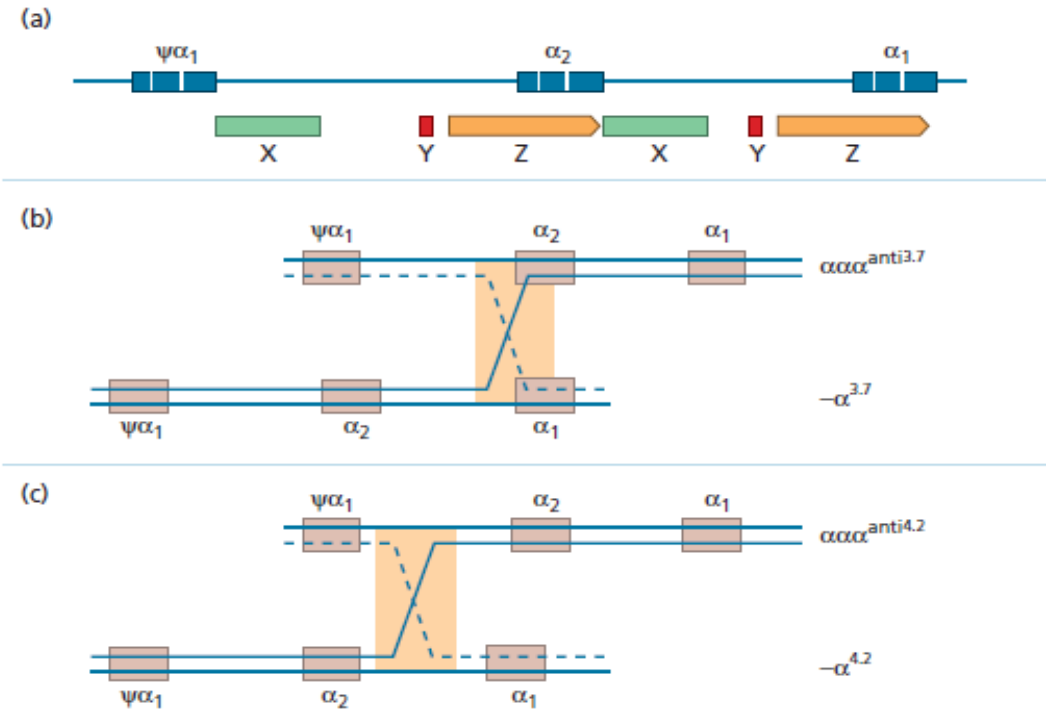

Figure 2.2. The molecular mechanism that underlie the deletion forms of α^{+} thalassaemia

(a) normal cluster showing X, Y and Z homology boxes

(b) 3.7-kb deletion

(c) 4.2-kb deletion

Adapted from Weather and Clegg 2001 (A. Victor Hoffbrand, 2016).

Another genetic problem that affects the α globin gene is point mutation that results in partially or completely inactivation of one of the α genes.

2.2.3 Pathophysiology of α thalassaemia

Clinically, for both forms of α thalassaemia can be broadly categorized into four syndromes and this depends on the number of functional α globin gene affected (as shown in Figure 2.3). Severe forms of α thalassaemia known as Hb Bart's hydrop fetalis or α thalassaemia major (--/--) and this syndrome occurs when all four genes are deleted or dysfunction. This syndrome is incompatible with extrauterine life (Harteveld and Higgs, 2010).

When only one functional α globin gene is inherited, it results in Hb H disease or also known as α thalassaemia intermedia (--/-α). This is usually the result of the compound heterozygous state for α^0 and α^+ thalassemia (Ahmad *et al.*, 2013). Patient with Hb H disease presented with variable severity of chronic haemolytic anaemia. It is characterized by greatly reduced rate of synthesis of the α chain and imbalance and excess of β chain production leads to the formation of an abnormal haemoglobin with β chain tetramers which referred to as Hb H. This Hb H is unstable and precipitates in erythroblasts and results in intramedullary cell death or also referred to as ineffective erythropoiesis (A. Victor Hoffbrand, 2016).

The third syndrome is α thalassaemia minor or also known as α thalassaemia trait. It is characterized by *cis* or *trans* positional loss of two α genes, resulting in heterozygosity for α^0 thalassemia (--/αα) and homozygosity for α^+ thalassemia (-α/-α). The individuals who inherit these genes defect usually are asymptomatic with mild hypochromic microcytic anaemia. Silent carriers with three functional genes (-α/αα) normally exhibit no clinical abnormalities with normal to mild changes in red cell indices (Ahmad *et al.*, 2013).

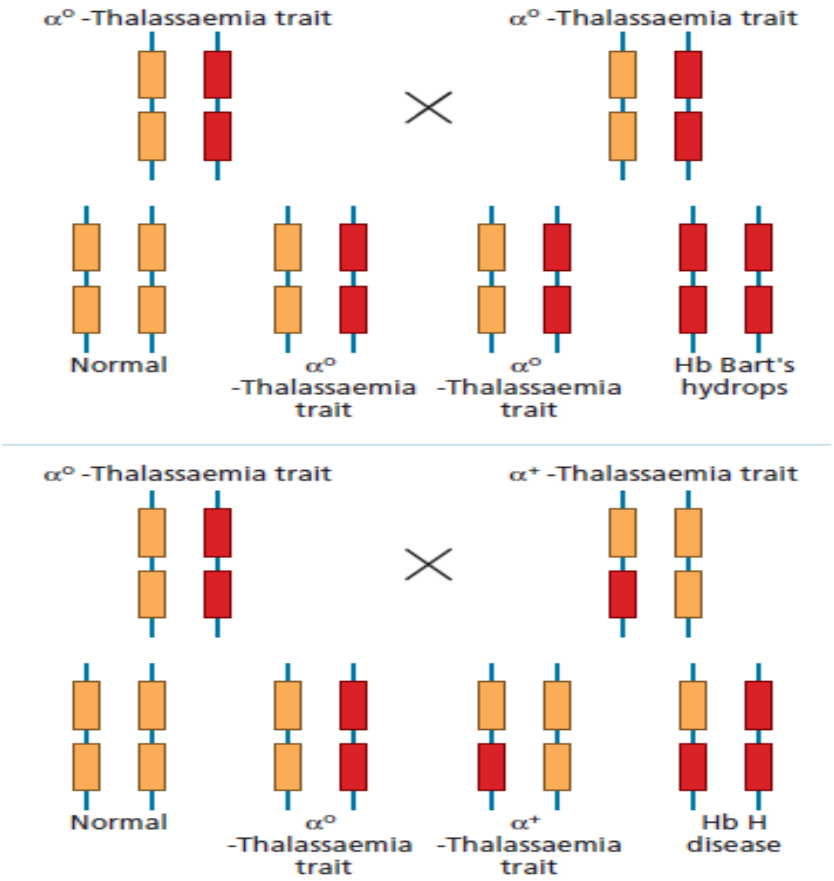

Figure 2.3. The genetics of α thalassaemia. Adapted from (A. Victor Hoffbrand, 2016).

Data from Malaysian Department of Statistics in 2010 showed that Malaysian population was about 28.3 million people. Malays and other Bumiputera comprised of 67.4% followed by Chinese, 24.6%, Indians 7.3% and others of 0.7% (Department of Statistics Malaysia, 2011). There is no new statistics data available onwards.

High prevalence of α thalassaemia was found in Malaysia and Southeast Asian countries. The gene frequency of α thalassaemia in Malaysia is 4.1% (Rahimah *et al.*, 2012). The gene frequencies in other neighbouring countries such as Indonesia were from 2.7-11% for α° and α^{+} thalassemia (Nainggolan *et al.*, 2003), Thailand of 20-30% (Kanokpongsakdi *et al.*, 2007) and Philipines of 2.6-3.2% (Ko *et al.*, 1999).

2.3 INTRODUCTION AND BACKGROUND OF HAEMOGLOBIN CONSTANT SPRING

Hb CS is one of the most prevalent non deletional α thalassaemia in Southeast Asia. Non deletional α thalassaemia can be broadly classified according to the type of mutations affected. Types of mutations described by Bain in 2006 are:

a. Ribonucleic acid (RNA) splice site mutation in α1 or α2 gene
b. RNA polyadenylation signal mutations
c. Impaired RNA translocation consequent on initiation codon or initiation consensus sequence mutation
d. Impaired RNA translation consequent on a frame shift or nonsense mutation
e. Impaired RNA translation consequent on a termination codon mutation leading to an elongated mRNA and α globin chain
f. Production of highly unstable α chain as a result of point mutation or a small deletion
g. Lack of transactivating factor encoded by the α thalassaemia mental retardation x-linked (ATRX) gene

Hb CS results from mutation of a termination codon at the α2 gene which leads to an elongated α chain. This added another 31 amino acids to the normal α-globin sequence. Mutation at the termination codon at the α2 globin gene results in unstable mRNA and produce an α thalassaemia 2- like effect. Mostly, the documented cases of non-deletional thalassaemia are often caused by mutation of the α2 gene than of the α1 gene. A patient with mutation in the α2 gene has more severe phenotype compared to those patient who inherit α2 gene deletions (Bain, 2006).

The mRNA of alpha Constant Spring (α^{CS}) only accounts for less than 1% of protein output of a normal α2 gene but it is very unstable compared with normal α mRNA. Fascinatingly, in terms of pathophysiology, the synthesis of even small amounts of elongated α^{CS} results in more severe anaemia than is seen in deletional α-thalassaemia. The mechanism behind this had been proposed that α^{CS} chains may have harmful effects on cellular and membrane properties of red blood cells with Hb CS (Bunn and Forget, 1986).

In 1997, Schrier *et al* studied the unusual pathobiology of Hb CS red blood cells. They found that the Hb CS-containing red blood cells were distinctly overhydrated relative to deletional α-thalassaemia variants. The derangement of volume regulation and cell hydration occured early in erythroid maturation and was fully expressed at the reticulocyte stage. They also found that the membrane rigidity and membrane mechanical stability of Hb CS-containing red blood cells was increased when compared with Hb H and α-thalassemia-1 trait red blood cells. They had suggested and concluded that the membrane pathology of Hb CS variants was caused by combination of the deleterious effects induced by membrane oxidized by α^{CS} and β globin chains (Schrier *et al.*, 1997).

Hb CS can be inherited as heterozygotes, homozygotes or combination with other thalassaemia variants and recognized as compound heterozygotes. The individuals in heterozygotes group are clinically and haematologically normal. Meanwhile, the homozygotes can resemble thalassaemia intermedia with mild anaemia and presence of hepatosplenomegaly. Interaction of Hb CS such as with α thalassaemia 1 deletion can lead to Hb H-CS disease ($-/\alpha^{CS}\alpha$). Individuals with Hb H-CS are clinically more severe than deletional Hb H disease. In some cases, the individuals can become transfusion dependent and also reported the incidence of Hb H-CS hydrops fetalis (Liao *et al.*, 2010).

The multi ethnic migrations and interactions can lead to interaction of Hb CS with other thalassaemia variants, such as co-inheritance of β thalassaemia or interaction of Hb CS with Hb E which is quite prevalent in Southeast Asian countries (Fucharoen *et al.*, 2003).

2.3.1 Prevalence and incidence of Haemoglobin Constant Spring

Hb CS is the most prevalent of mutational α thalassaemia in Southeast Asian countries. The gene frequencies of Hb CS vary between 1 to 8% (Fucharoen and Winichagoon, 1992).

Rahimah *et al* in 2013 had collected data from the Insitute of Medical Research (IMR) regarding the distribution of α thalassaemia gene variants in diverse ethnic populations in Malaysia. They retrieved the haematological and molecular data from more than 5000 patients referred from all hospitals and centres in Malaysia for α thalassaemia screening to IMR from year 2007 till 2010.

Three common non deletional mutations identified were Hb Adana, Hb Quong Sze and Hb CS. Hb CS is the most common non deletional thalassaemia compared to the other two types of mutational α thalassaemia identified. In Malays and Sabahans, it is the third common α thalassaemia determinants. Orang Asli aborigines recorded the highest incidence of Hb CS with incidence of 11.5% meanwhile the Malaysian Chinese recorded the lowest incidence of 0.7% (Ahmad *et al.*, 2013).

2.4 LABORATORY TECHNIQUES FOR THE IDENTIFICATION OF THALASSAEMIA/ HAEMOGLOBINOPATHIES

To diagnose thalassaemia and haemoglobinopathies, it requires combination of laboratory techniques. At least two techniques must be done with the assessment of the results must correlated with the clinical findings and background ethnicity of the patients (Bain, 2006).

Venous blood will be taken and collected in ethylene diamine tetra-acetic acid (EDTA) as anticoagulation. It should be stored at temperature of 4°C and should be tested within a week. Storage longer than one week will affect the result as it will lead to denaturation of haemoglobin. Request for haemoglobin analysis for thalassaemia must be accompanied by proper identification name, age, ethnicity, clinical information as well as family history (Bain, 2006).

International Committee for Standardization in Haematology expert panel on thalassaemia and haemoglobinopathy had made recommendations for diagnostic laboratory investigations for these conditions. Initial tests recommended were full blood count (FBC), haemoglobin electrophoresis at alkaline pH, sickling test and quantification of abnormal Hb A_2 and Hb F (Clarke and Higgins, 2000).

2.4.1 Full blood count and peripheral blood film

Initially, samples for haemoglobin analysis for thalassaemia either α or β or haemoglobin variants must be tested for FBC and smear must be done to check for red cell morphology. If a full blood picture shows polychromasia, a reticulocyte count is indicated. Other than that, FBC is initially done for thalassaemia screening (Ministry of Health Malaysia, 2009).

The thalassaemia screening is done to identify carriers of thalassaemia or haemoglobinopathy disorders. Pre test counselling is carried out for both groups (voluntary or index case group) before the FBC is taken. The level of mean cell haemoglobin (MCH) of <27 pg had been chosen before subjected the individuals for further test such as haemoglobin analysis (Ministry of Health Malaysia, 2009).

MCH is preferably selected as a cut-off point for thalassaemia screening as the parameter is less susceptible to storage changes compared to mean cell volume (MCV). MCV is also raised by a number of conditions such as having concurrent vitamin B12 or folate deficiencies or in HIV patients who is taking nucleoside analogues due to impaired deoxyribonucleic acid (DNA) synthesis (Howard *et al.*, 2005).

Other parameter in FBC that can help in distinguishing thalassaemia from nutritional anaemia (such as iron deficiency anaemia) as the latter also can present with hypochromic microcytic anaemia is red cell distribution width (RDW). RDW is a measure of the degree of variation in red cell size and may provide useful information to support the diagnosis but cannot be used as a single indicator. In iron deficiency anaemia, the RDW is increased as presence of anisopoikilocytosis while in thalassaemia, it produces uniform microcytic red cells, thus the RDW is not increased (Clarke and Higgins, 2000).

The haemoglobin cannot be used as a sole indicator to diagnose thalassaemia and haemoglobinopathy as in haemoglobin variant or minor α thalassaemia, the individuals can have normal or slightly low haemoglobin (Clarke and Higgins, 2000).

In Thailand, a study had been done to evaluate the effectiveness of the automated haematology analyzer for thalassaemia screening using URIT-2900 haematology analyzer. The suitable cut off MCV and MCH had been determined in which 78 fl and 27pg respectively for screening of α°thalassaemia, β thalassaemia and Hb E (Karnpean *et al.*, 2011).

Full blood picture can also give clue in aiding the diagnosis of thalassaemia and haemoglobinopathy. In case of beta (β) thalassaemia, there is hypochromic microcytic picture with anisopoikilocytosis. Meanwhile in Hb CS particularly in heterozygotes group, the individuals might have mild hypochromic microcytic anaemia and some can have normal red cell parameters (Bain, 2006).

2.4.2 Haemoglobin H inclusion test (brilliant cresyl blue test or new methylene blue test)

This is a technique for the demonstration of Hb H inclusions when there is a suspicion of Hb H disease or when there is presence of H band on the haemoglobin electrophoresis. Brilliant cresyl blue is an oxidant in which it will precipitates in the red cells when it is mixed with the Hb H. Hb H is unstable and when it precipitates it gives the small blue staining inclusion that is evenly distributed and gives the appearance of golf ball appearance (Hartwell *et al.*, 2005) (as shown in Figure 2.4).

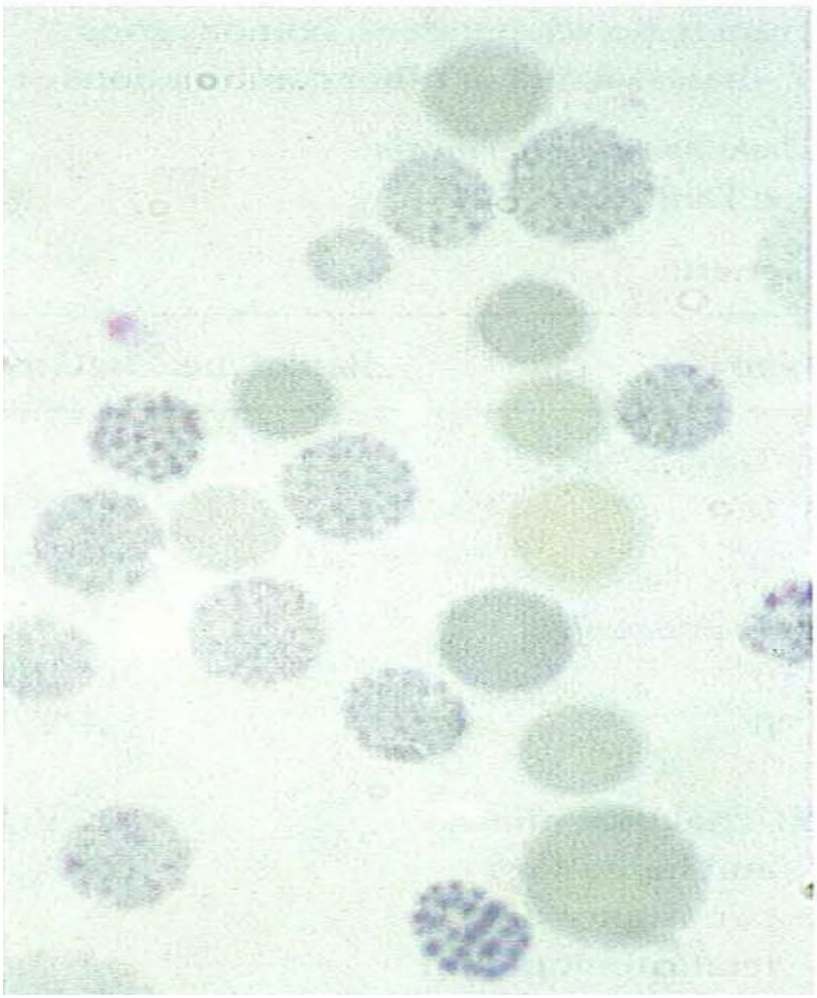

Figure 2.4. Haemoglobin Inclusion test showing golf ball appearance. Adapted from (Moss, 2011).

2.4.3 Haemoglobin electrophoresis

Haemoglobin electrophoresis is still the most common technique for initial detection of thalassaemia and haemoglobinopathies although new modalities are available such as high performance liquid chromatography (HPLC) or CE (Ager and Lehmann, 1958).

It is carried out on a filter paper, a cellulose acetate membrane at alkaline pH or citrate or agarose gel at acidic pH. It is must be performed on lysed packed cells and not whole blood as the latter will have interference with presence of paraprotein or high concentration of

polyclonal immunoglobulins, in which can lead to a prominent band which can be confused with haemoglobin variant (Bain, 2006).

The principles of haemoglobin electrophoresis is based on when the proteins are applied to a membrane and exposed to charge gradient, the band separated from each other and the separation of the bands can be visualized by staining with either haem or protein stain. Initially the haemoglobin electrophoresis will be done at alkaline pH as it will detect the presence of Hb A, S/D/G/ Lepore, F, A_2,C,E,O-Arab and H as well Hb CS. Each test must be run together with control samples containing Hb A,S,F and C (Bain, 2006).

Hb CS is unstable haemoglobin, therefore there is no specific band on haemoglobin electrophoresis as it can present at any areas. If variant haemoglobin is detected after initial test with haemoglobin electrophoresis at alkaline pH, it is essential to confirm the variant by other technique such as running the haemoglobin electrophoresis at an acidic pH by using citrate or agarose gel. Other modalities to confirm the presence of abnormalities also must be performed by either using HPLC or CE (Clarke and Higgins, 2000).

2.4.4 High performance liquid chromatography (HPLC)

To detect haemoglobin abnormalities, HPLC is a sensitive and precise method (Ou *et al.*, 1983). HPLC is one of the available methods of choice for initial screening for haemoglobin variants which is also suitable for neonatal screening as it can quantify Hb A, Hb A_2 and Hb F (Eastman *et al.*, 1996).

HPLC utilises a weak cation exchange column in which it is a process of cumulation of molecules (normal and variant hemoglobins) and separation into different fractions by their adsorption onto a negatively charged stationary phase in a chromatography column, followed by their elution by a mobile phase. The mobile phase contains liquid with incrementing concentrations of cations flowing through the column. The cations will compete with the adsorbed proteins for the anionic binding sites. As a result, the adsorbed positively charged haemoglobin molecules are eluted from the column at a rate cognate to their affinity in the stationary phase into the liquid phase (Bain, 2006).

Different types of haemoglobin give peaks at a particular retention time. The area under the retention time are quantified and plotted on the chromatogram. It is an automated system and is primarily indicated for the measurement of HbA_2 and HbF but it also provides retention times for several haemoglobin variants (Brants, 2011).

Although many haemoglobin variants can be separated from each other, some of the variant can overlap with each others. The advantages of using HPLC compared to the haemoglobin electrophoresis are it is an automated system and rapid with high precision. It is less labour intensive and required small sample to run with. Moreover, it can quantify normal and variant haemoglobins such as in case of β thalassaemia carier, it can quantify percentage of Hb A_2 level (George *et al.*, 2001).

The disadvantages of using HPLC are greater reagent cost and it requires technical expertise. Furthermore, variant haemoglobin may have same retention times as normal haemoglobin. For example, Hb Lepore may overlap with Hb A_2 on the chromatogram. Hb A_2 also cannot be accurately quantified in the presence of Hb S and Hb E as a result of glycated Hb S and E

co-eluting with Hb A_2. However, HPLC accurately quantifies Hb A_2 in the presence of Hb C (Greene *et al.*, 2012).

Hb Bart and Hb H elute in the void volume (pre-calibration peak) and only can be distinguished when the levels are higher than 5%. Certain artefacts such as bilirubin also can interfere in the void volume and may lead to a sharp peak in the same retention area as Hb H, Hb Bart and acetylated Hb F. Hb CS elute in the Hb C window with the retention time of 4.90-5.30. Hb O-Arab also elute in the same window (Greene *et al.*, 2012). Hb CS gives a very small peak at the C window and sometimes can be missed (Pornprasert *et al.*, 2015).

2.4.5 Capillary electrophoresis (CE)

2.4.5 (a) History

Hjerten in 1967 was the first person who described electrophoretic separation of molecules in a glass tube and subsequent detection of the separated components by using ultraviolet absorption (Hjertén, 1967). The idea was unpopular until in 1981, Jorgenson and Lucaks introduced the high resolution power of capillary zone electrophoresis (Jorgenson and Lukacs, 1981). In years afterwards, many advances have applied the principles such as systemic analysis of proteins, genomics as well as for thalassaemia and haemoglobinopathies. The CE system was approved by USA Food and Drug Administration in 2007 for the evaluation of haemoglobinopathies (Keren *et al.*, 2008).

2.4.5 (b) Principles, background and results

All CE techniques are carried out using similar apparatus (Bosserhoff and Hellerbrand, 2005):

- A high voltage power supply
- The anode and cathode buffer reservoirs
- The separation chamber (capillary tube)
- The injection system
- The detector

For analysis of thalassaemia and haemoglobinopathies, it uses similar principles as other CE, by using free solution. Charged molecules are separated by their electrophoretic mobility in alkaline buffer with a specific pH. Other separation factors are electrolyte pH and and electroosmotic flow. It is an automated analyzer in which the assay is performed on the haemolysate of whole blood samples that had been collected in EDTA tubes as an anticoagulant. A complete haemoglobin profile is conducted in which it quantifies fractions of normal haemoglobin (Hb A, A_2 and F) as well as detects the major haemoglobin variants such as Hb C,E ,S and D (Sebia, 2013).

The Capillarys 2 Flex Piercing instruments can allow 8 simultaneous analyses functioning in parallel. First, a sample is diluted with haemolysing solution and by aspiration; it is injected at the anodic end of capillary. Protein separations at a high voltage is then performed and direct detection of haemoglobin is made at the cathodic end of the capillary at an absorbance wave length of 415 nm. The resulting electrophoregrams are evaluated visually for pattern abnormalities (Sebia, 2013).

Different haemoglobin variants are identified in zones Z1 to Z15. Hb A_2 is identified in Zone 3 while Hb A is found in Zone 9 (as shown in Figure 2.5). For Hb CS, it is identified in Zone 2 of capillary electrophoresis or also known as Zone C (as shown in Figure 2.6). Other haemoglobin variants that share the same position as Hb CS are Hb Paksé (which is reported to be found in Thailand), Hb C, Hb A_2 variant and other types of rare haemoglobin variants.

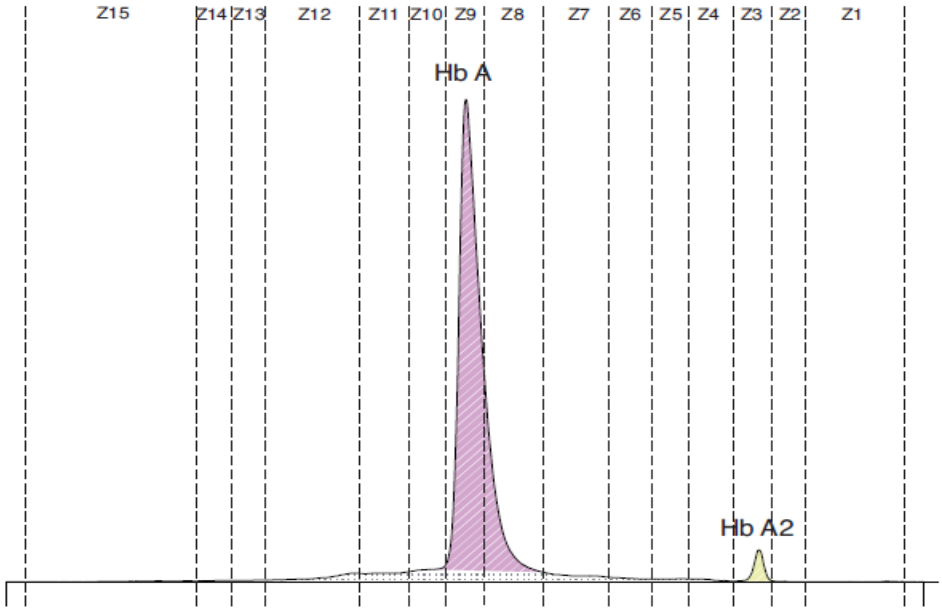

Figure 2.5. Electrophoregram of normal sample: normal haemoglobin fractions. Adapted from Manual of Capillarys Haemoglobin (E) using the Capillarys 2 Flex-Piercing Instrument, 2013/01.

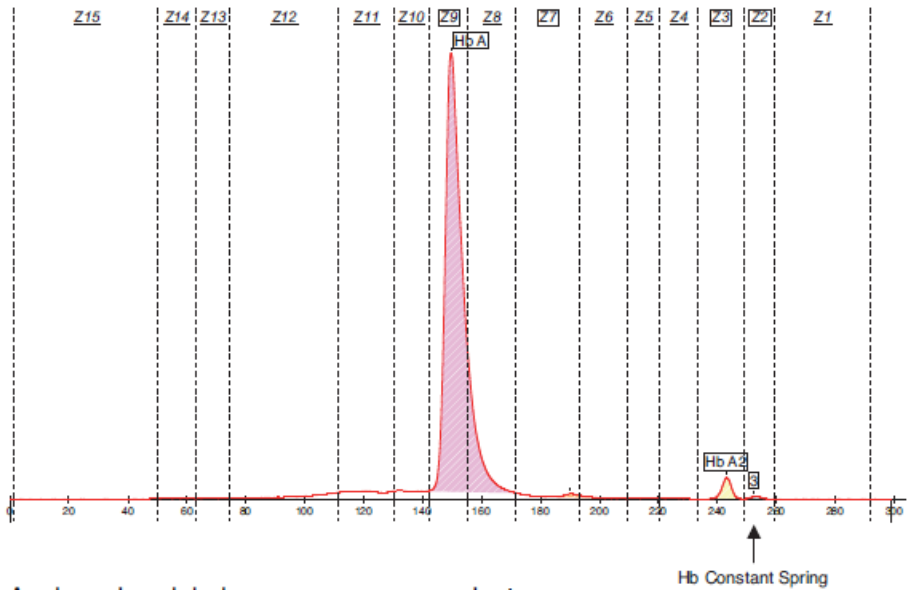

Figure 2.6. Electrophoregram of Hb CS at Zone 2 of capillary electrophoresis. Adapted from Manual of Capillarys Haemoglobin (E) using the Capillarys 2 Flex-Piercing Instrument, 2013/01.

Appropriate sample collection and storage is very important as it can interfere with the results. Fresh anticoagulated whole blood samples collected in EDTA bottle are recommended. The samples should only be stored up to seven days between 2°C to 8°C. The reason behind this is progressive degradation of haemoglobin might occur. Apart from that, when the age of sample is longer than seven days, it may interfere with the results. For example, "aging fraction" of Hb A will appear more anodic, which is in Zone 11 despite the usual zone identified which is in Zone 9 (Sebia, 2013).

Suwannakan *et al* in 2011 had published a multicentre validation of fully automated CE for diagnosis of thalassaemia and haemoglobinopathies in Thailand. It was conducted in four different reference laboratories. The performance characteristics including precision and accuracy had been analysed and compared with existing validated HPLC and automated cation exchange low pressure liquid chromatography (LPLC). Comparison of the three methods performed to assess accuracy of Hb A_2 and F showed good linear correlation. It